

Inhibition of Insulin Sensitivity by Free Fatty Acids Requires Activation of Multiple Serine Kinases in 3T3-L1 Adipocytes

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Insulin receptor substrate (IRS) has been suggested as a molecular target of free fatty acids (FFAs) for insulin resistance. However, the signaling pathways by which FFAs lead to the inhibition of IRS function remain to be established. In this study, we explored the FFA-signaling pathway that contributes to serine phosphorylation and degradation of IRS-1 in adipocytes and in dietary obese mice. Linoleic acid, an FFA used in this study, resulted in a reduction in insulin-induced glucose uptake in 3T3-L1 adipocytes. This mimics insulin resistance induced by high-fat diet in C57BL/6J mice. The reduction in glucose uptake is associated with a decrease in IRS-1, but not IRS-2 or GLUT4 protein abundance. Decrease in IRS-1 protein was preceded by IRS-1 (serine 307) phosphorylation that was catalyzed by serine kinases inhibitor κ B kinase (IKK) and c-JUN NH₂-terminal kinase (JNK). IKK and JNK were activated by lino-

leic acid and inhibition of the two kinases led to prevention of IRS-1 reduction. We demonstrate that protein kinase C (PKC) θ is expressed in adipocytes. In 3T3-L1 adipocytes and fat tissue, PKC θ was activated by fatty acids as indicated by its phosphorylation status, and by its protein level, respectively. Activation of PKC θ contributes to IKK and JNK activation as inhibition of PKC θ by calphostin C blocked activation of the latter kinases. Inhibition of either PKC θ or IKK plus JNK by chemical inhibitors resulted in protection of IRS-1 function and insulin sensitivity in 3T3-L1 adipocytes. These data suggest that: 1) activation of PKC θ contributes to IKK and JNK activation by FFAs; 2) IKK and JNK mediate PKC θ signals for IRS-1 serine phosphorylation and degradation; and 3) this molecular mechanism may be responsible for insulin resistance associated with hyperlipidemia. (*Molecular Endocrinology* 18: 2024–2034, 2004)

IT IS ESTIMATED that there were 11 million diabetic patients (prevalence 4.0%) in the United States in the year 2000, and this number is going to increase to 29 million (prevalence 7.2%) by 2050 (1). Type 2 diabetes accounts about 95% of the total diabetic cases. Although it is known that type 2 diabetes is closely associated with obesity, it remains to be investigated how obesity leads to type 2 diabetes. It is generally believed that insulin resistance is a major risk factor for type 2 diabetes. Three possible mechanisms have been suggested for the pathogenesis of insulin resistance. The first is that obesity leads to hyperlipidemia. A high level of free fatty acids (FFAs) in the plasma

induces insulin resistance (2, 3). The second is that obesity results in overproduction of insulin-desensitizing cytokines including TNF- α , and TNF- α contributes to insulin resistance (4). The third is that obesity increases activity of protein tyrosine phosphatases that interrupt insulin signaling by dephosphorylating the insulin receptor substrate (IRS) (5).

It has been known for more than a decade that FFAs can induce insulin resistance (6). In human (6) or animals (7), hyperlipidemia generated by iv infusion of lipid/heparin consistently induces acute insulin resistance in the body. The glucose tolerance returns to the normal range after hyperlipidemia is eliminated. At the molecular level, IRS protein has been suggested as a target of FFAs for insulin resistance (7, 8). Phosphorylation of serine 307 (Ser307) in IRS-1 protein has been linked to FFA-associated insulin resistance (7). In the normal rats, infusion of a lipid emulsion results in IRS-1 Ser307 phosphorylation, and this phosphorylation correlates to a reduced PI(3)K (phosphatidylinositol-3 kinase) activity in the skeletal muscle (7). However, it is not clear how FFAs lead to Ser307 phosphorylation in IRS-1.

Abbreviations: 15dPGJ₂, 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂; FFAs, free fatty acids; HA, hemagglutinin; IKK, inhibitor κ B kinase; IP, immunoprecipitation; IRS-1, insulin receptor substrate 1; JNK, c-JUN NH₂-terminal kinase; PI(3)K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PKD, protein kinase D; Ser307, serine 307.

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IRS-1 Ser307 phosphorylation is inducible and responsible for the inhibition of IRS-1 function (9–12). White's group first demonstrates that IRS-1 Ser307 phosphorylation is induced by stimuli that lead to c-JUN NH₂-terminal kinase (JNK) activation (9, 13). Ser307 phosphorylation may lead to the inhibition of IRS-1 function through interrupting IRS/insulin receptor interaction (10) or promoting protein degradation of IRS-1 (14). Our previous studies suggest that in addition to JNK, inhibitor κ B kinase (IKK) also phosphorylates Ser307 (Ser312 in the human IRS-1) in IRS-1 protein in response to TNF- α or serine phosphatase inhibitor calyculin A (11, 12).

In this study, we investigated the molecular events underlying FFA-induced insulin resistance. We observed that linoleic acid induced insulin resistance in 3T3-L1 adipocytes. This cellular model reflects insulin resistance induced by high-fat diet in C57BL/6J mice. The insulin resistance was associated with a Ser307 phosphorylation followed by IRS-1 protein reduction. Activation of IKK and JNK was induced by FFA, and activities of the two serine kinases were required for Ser307 phosphorylation and degradation of IRS-1. We show that protein kinase C (PKC) θ is expressed in fat tissue and activation of PKC θ by FFA leads to induction of IKK and JNK activities.

RESULTS

Linoleic Acid Induces Insulin Resistance

We used 3T3-L1 adipocytes as a cellular model analyzing FFA signaling pathway. 3T3-L1 adipocytes were

treated with linoleic acid (C18 $\Delta^{9,12}$) to induce insulin resistance. Insulin-induced glucose uptake was measured to determine insulin sensitivity. The result shows that insulin-induced glucose uptake was inhibited by as much as 70% after a 16 h-treatment with linoleic acid (Fig. 1A). This is consistent with reports that FFA induces insulin resistance in cell culture (8, 15). To understand the role of IRS-1 in the mechanism of FFA-induced insulin resistance, IRS-1 protein abundance was monitored in the FFA-treated cells in a time-course study (Fig. 1B). IRS-1 protein decreased gradually. A 50% decrease was detected 6 h after addition of linoleic acid. At 24 h, 80% of IRS-1 protein was lost. Because IRS-1 protein abundance is mainly regulated by protein degradation (16–18), this result suggests that the reduction in IRS-1 protein may be a result of protein degradation in 3T3-L1 adipocytes.

IRS-2 and GLUT4 proteins were not reduced (Fig. 1, B and C). Interestingly, GLUT1 was induced by FFA (Fig. 1C). These results suggest that inhibition of insulin-induced glucose uptake by FFA is not a result of reduction in IRS-2 or GLUT4. It is known that GLUT1 does not involve in insulin-induced glucose uptake.

FFA Induces Serine Phosphorylation of IRS-1

Because serine phosphorylation precedes IRS-1 degradation (16–18), our results suggest that FFA may induce IRS-1 serine phosphorylation. To test the hypothesis, 3T3-L1 adipocytes were treated with linoleic acid and IRS-1 phosphorylation was determined with the phospho-specific IRS-1 (Ser307) antibody by immunoblotting. The phosphorylation was induced by linoleic acid, and the induction was in a dose- and

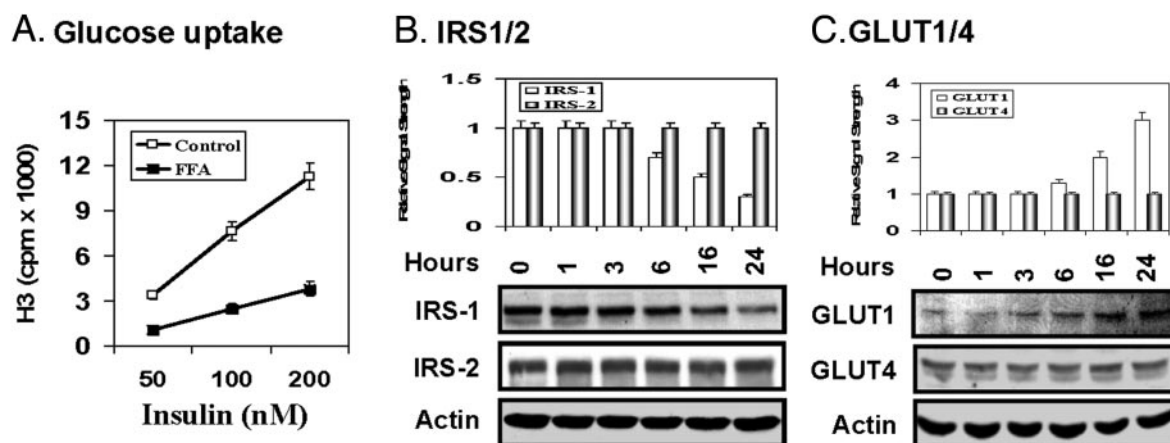


Fig. 1. Insulin Resistance Induced by Fat Acids

Fully differentiated 3T3-L1 adipocytes were treated with BSA-bound linoleic acid (300 μ M) in serum-free medium. Glucose uptake was determined 16 h later with different dose of insulin as indicated. A, Insulin-induced glucose uptake. Each bar represents mean \pm SE of results from triplicates. The experiment was repeated three times with consistent results. B, IRS-1 reduction in 3T3-L1 adipocytes. IRS-1 and IRS-2 proteins were determined in the whole cell lysate by immunoblotting. C, GLUT1 and GLUT4 in 3T3-L1 adipocytes. GLUT1 and GLUT4 proteins were determined in the whole cell lysate by immunoblotting. In panels B and C, signals were quantified, and each bar represents mean \pm SE of results from three independent experiments. The representative blot is shown.

time-dependent manner. Linoleic acid was able to promote the phosphorylation at 100 μM and the strongest activity was observed at 300 μM (Fig. 2A). When FFA dose was further increased, the phosphorylation was reduced at 400 μM . FFA BSA does not have this activity (data not shown). In the time-course study, a 300 μM concentration of linoleic acid was used to treat the cells for different times (Fig. 2B). IRS-1 phosphorylation was increased at 1 h, and the signal was maintained up to 5 h before a drop at 8 h. Stearic (C18) and oleic (C18 Δ^9) acids were compared with linoleic acid for induction of IRS-1 phosphorylation (Fig. 2C). There is no significant difference in these FFAs (C18, C18 Δ^9 , and C18 $\Delta^{9,12}$) as indicated by IRS-1 Ser307 phosphorylation. This suggests that saturation status of FFAs may not play a role in the serine phosphorylation of IRS-1.

IKK and JNK Mediate FFA-Induced IRS-1 (Ser307) Phosphorylation

Ser307 of IRS-1 can be phosphorylated by either IKK or JNK (10, 12). Activation of these two serine kinases was examined after FFA treatment using the phospho-specific IKK or JNK antibodies. In mammalian cells, phosphorylation of Ser181 at the activation loop is essential for activation of the catalytic activity of IKK (19, 20). Similarly, phosphorylation of Thr183 and Tyr185 are required for activation of JNK (21). For a 5-h treatment with linoleic acid, phosphorylation of IKK (Ser181) and JNK2 (Thr183/Tyr185) were both increased in 3T3-L1 adipocytes (Fig. 3A). However, activation of the two kinases exhibited a difference in dose-dependence. IKK activation was observed at 100 μM and JNK2 activation was detected at 300 μM (Fig. 3A), suggesting that IKK is more sensitive to FFAs. It seems that JNK1 is constitutively activated in 3T3-L1 adipocytes and JNK2 activity is induced by FFA. To confirm the role of IKK and JNK in Ser307 phosphorylation of IRS-1, specific inhibitors 15-deoxy- $\Delta^{12,14}$ -prosta-

glandin J₂ (15d-PGJ₂) and SP600125 were used to inhibit IKK and JNK, respectively (22, 23). As expected, inhibition of IKK resulted in a reduction in Ser307 phosphorylation (Fig. 3B). Similarly, inhibition of JNK by SP600125 also blocked Ser307 phosphorylation (Fig. 3C). These data are consistent with those observed in HepG2 and 3T3-L1 preadipocytes that IKK and JNK mediate Ser307 phosphorylation (11, 12).

PKC Activation by Linoleic Acid

The molecular events underlying IKK and JNK activation by FFAs is largely unknown. PKC is suggested as a kinase that mediates FFA-induced signals for insulin resistance in the skeletal muscle (7, 24–26). It is not clear whether PKC mediates FFA signal in adipocytes. In this study, we analyzed phosphorylation status of different PKC isoforms in adipocytes to determine their activation. It is known that the catalytic activity of PKC is associated with phosphorylation of serine/threonine at the activation and autophosphorylation domains (27–29). Phosphorylation of PKC was determined in immunoblot using phospho-specific antibodies. The result shows that phosphorylation of PKC θ (Thr538) and PKC ζ (Thr410) was induced by linoleic acid (Fig. 4, A and B). Phosphorylation of PKC θ exhibited a peak at 200 μM of the FFA. PKC ζ phosphorylation reached the peak at 400 μM . Functional consequence of PKC activation is indicated by phosphorylation of the downstream substrate protein kinase D (PKD) (also known as PKC μ) (30, 31). Phosphorylation of PKD at Ser744/748 is dependent on PKC activity (31) and is increased in a similar pattern to that of PKC θ (Fig. 4, A and B). Phosphorylation of PKC α/β (Thr638/641), and PKC δ (Ser643) was not changed by FFA in 3T3-L1 adipocytes (data not shown). These results suggest that PKC θ and PKC ζ are activated by FFA in 3T3-L1 adipocytes.

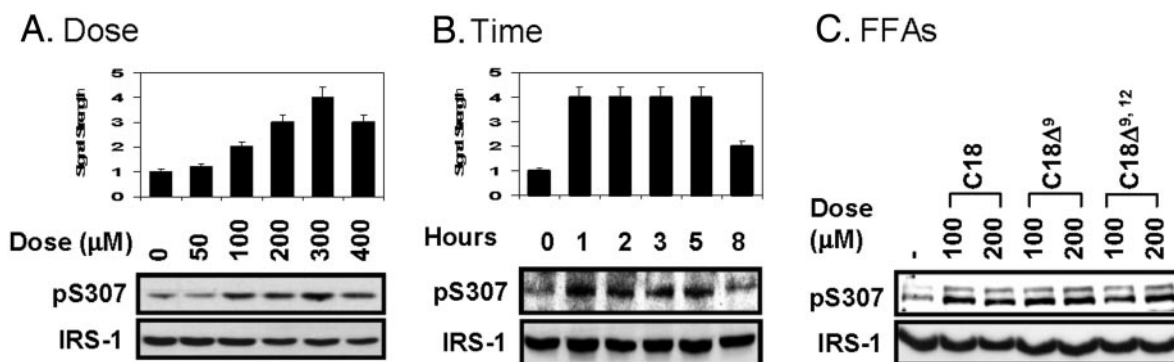


Fig. 2. Induction of Ser307 Phosphorylation by FFAs

3T3-L1 adipocytes were treated with BSA-bound FFAs at different doses or a fixed dose (300 μM) for different times as indicated. IRS-1 phosphorylation was determined in the whole cell lysate by immunoblotting with the phospho-specific IRS-1 (Ser307) antibody. All the experiments were repeated three times with consistent results. Each bar represents mean \pm SE of results from three experiments in the bar figure. A representative Western blot is shown in each panel. A, Dose response. The cells were treated with linoleic acid for 5 h. B, Time course. The cells were treated with 300 μM of linoleic acid. C, Comparison of fatty acids. The cells were treated with stearic acid (C18), oleic acid (C18 Δ^9) and linoleic acid (C18 $\Delta^{9,12}$) for 5 h.

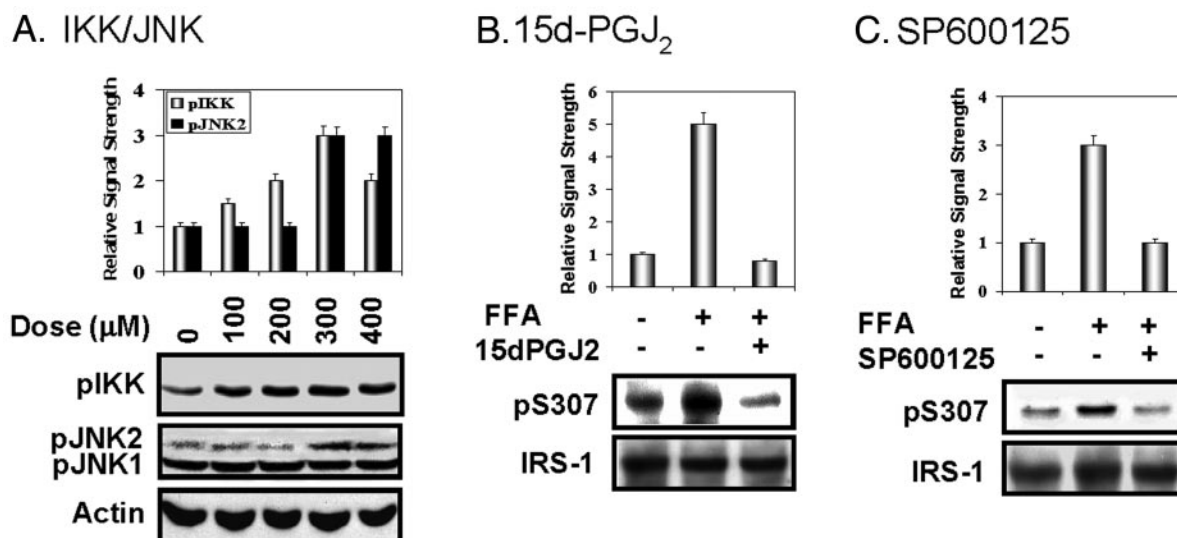


Fig. 3. Activation of IKK and JNK by FFAs

3T3-L1 adipocytes were treated with linoleic acid at different doses. IKK and JNK activation was then determined in the whole cell lysate by immunoblotting with phospho-specific antibodies. The experiments were repeated three times with consistent results. Each bar represents mean \pm SE of results from three experiments in the bar figure. A representative Western blot is shown in each panel. A, Dose response of IKK and JNK activation in linoleic acid treatment for 5 h. B, Inhibition of IKK. 3T3-L1 adipocytes were pretreated with IKK inhibitor 15dPGJ₂ (15 μ M, 30 min) to inhibit IKK activity. C, Inhibition of JNK. 3T3-L1 adipocytes were pretreated with JNK inhibitor SP600125 (30 μ M, 30 min). In B and C, the cells were treated with 300 μ M of linoleic acid for 3 h to induce Ser307 phosphorylation.

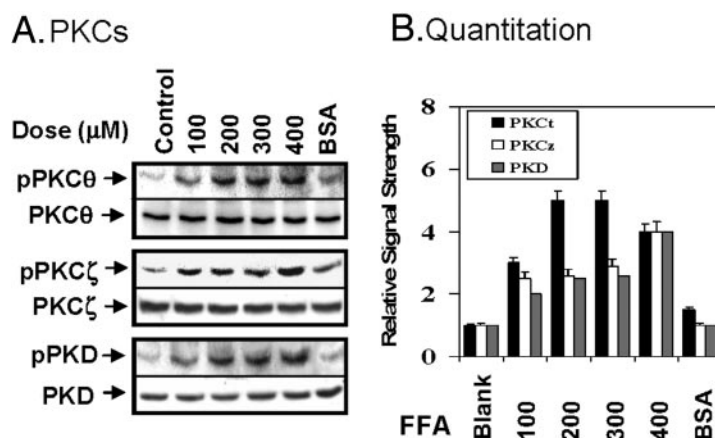


Fig. 4. Activation of PKC θ by FFA

3T3-L1 adipocytes were treated with linoleic acid at different doses for 5 h. Activation of PKC isoforms was then determined with phospho-specific antibodies. Phospho-specific antibodies to PKC θ (Thr538), PKC ζ (Thr410), and PKD (Ser744/748) were obtained from Cell Signaling and used in the immunoblotting. The experiments were conducted by immunoprecipitating PKC with isoform specific antibodies followed by immunoblotting with phospho-specific antibodies. The experiment was repeated three times with consistent results. A, Phosphorylation of PKCs. B, Quantitation of signals in panel A. Each bar represents a mean \pm standard error of results from three measurements.

Inhibition of PKC

A role of PKC in the induction of Ser307 phosphorylation has been reported recently (32). However, it is not clear how PKC leads to IRS-1 serine phosphorylation because PKC has not been shown to phosphorylate Ser307 directly. It is likely that IKK and JNK mediate PKC activity because it is known

that PKC can activate IKK and JNK. To test the possibility, we used a PKC-specific inhibitor calphostin C (33). Calphostin C inhibits conventional and novel PKCs through competition with diacylglycerol at the regulatory domain of PKC (33). Calphostin C exhibited a strong inhibitory effect on IRS-1 Ser307 phosphorylation in cells treated with PKC activator (phorbol 12-myristate 13-acetate)

(Fig. 5A). Calphostin C also inhibited FFA-induced phosphorylation of IKK and JNK (Fig. 5B), suggesting that IKK and JNK activation is dependent on PKC activity. Consistently, IRS-1 (Ser307) phosphorylation was also inhibited by calphostin C in FFA-treated adipocytes (Fig. 5C). These results suggest that IKK and JNK mediate PKC activity in IRS-1 (Ser307) phosphorylation in adipocytes. Because PKC ζ is not sensitive to Calphostin C, the result does not support that PKC ζ is involved in IRS-1 Ser307 phosphorylation induced by FFAs.

Impairment of IRS-1 Interaction with Up- and Downstream Signaling Component by Ser307 Phosphorylation

The above observations suggest that Ser307 phosphorylation is responsible for the impairment of IRS-1 function. To test this possibility, insulin-induced association of IRS-1 and insulin receptor was examined using the hemagglutinin (HA)-tagged recombinant human IRS-1. To determine the role of Ser307, point mutation was introduced into IRS-1 protein to replace Ser307 with either alanine (A) or aspartate (D). The recombinant IRS-1 was expressed in NIH3T3 cells that express insulin receptor through stable transfection. The association of IRS-1 and insulin receptor was examined by immunoprecipitation (IP) with a monoclonal antibody to the insulin receptor. The result shows that the “A” mutant (IRS-1A) that represents an unphosphorylated Ser307 exhibited an affinity to insulin receptor (Fig. 6A). The “D” mutant (IRS-1D) that mimics the phosphorylated Ser307 was unable to associate with the insulin receptor. Expression of different forms of the recombinant IRS-1 is consistent in the transfected cell (Fig. 6A). These data support that phosphorylation of Ser307 leads to an impairment of IRS-1/IR interaction. To confirm this effect, phosphorylation of IRS-1 at tyrosine 632 (Y632) was examined by immunoblotting of IRS-1 with pY632-specific antibody in the IP product. The phosphorylation was induced by insulin in 5 min. Exposure to linoleic acid led to a reduction in Y632 phosphorylation (Fig. 6B). This

reduction was detected in 3 h of FFA treatment, suggesting that the inhibition of IRS-1 function is dependent on duration of FFA treatment. This time point is consistent with that observed *in vivo* for lipid-induced insulin resistance (34).

IRS-1 function was also examined by determining association of IRS-1 with p85 of PI(3)K and by Akt phosphorylation. 3T3-L1 adipocytes were treated with linoleic acid for different times to induce Ser307 phosphorylation, and the IRS-1/p85 association was induced by insulin. The association was determined in IP with IRS-1 antibody. A reduction in p85 abundance was observed after FFA treatment, and this became detectable at 3 h with FFA treatment (Fig. 6C). Consistently, activation of Akt was also inhibited as indicated by Thr308 phosphorylation (Fig. 6D). These results further support that serine phosphorylation of IRS-1 leads to impairment of PI(3)K signal transduction.

Restoration of Insulin Sensitivity by PKC Inhibitor

Pharmacological inhibitors of PKC, IKK, and JNK were tested to rescue IRS-1 protein from degradation. PKC inhibitor Calphostin C or combination of IKK and JNK inhibitors was used to pretreat 3T3-L1 adipocytes. IRS-1 protein abundance and glucose uptake were examined in 3T3-L1 adipocytes 16 h later after FFA-treatment. The results show that the inhibitors are able to block IRS-1 degradation completely (Fig. 7A). The inhibitors also protected insulin-induced glucose uptake in adipocytes (Fig. 7B). After pretreatment with the inhibitors, the glucose uptake was significantly restored in the FFA-treated cells. It is noted that the restoration was not complete. This may be a result of limitation of the inhibitor activity.

PKC θ in the Adipose Tissue of Dietary Obese C57BL/6J Mice

Although PKC θ can be activated by FFA in the skeletal muscle (7, 24, 26, 35, 36), it has not been reported whether this happens in the adipose tissue. To ad-

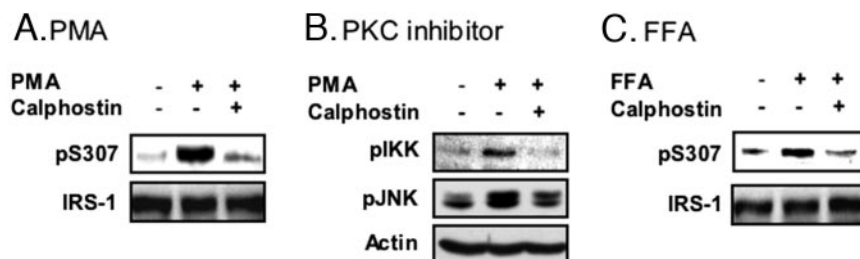


Fig. 5. Inhibition of PKC

Calphostin C (0.1 μ M) was used to pretreat the 3T3-L1 adipocytes for 30 min. After treatment with phorbol 12-myristate 13-acetate (PMA) or linoleic acid for 3 h, the whole cell lysate was subjected to analysis by immunoblotting with antibodies as indicated. All the experiments were repeated three times with consistent results. A representative immunoblot is shown in each panel. A, PKC-specific inhibitor calphostin C was used to block PMA (100 nM for 1 h) induced PKC activities. B, Inhibition of PMA-induced IKK and JNK activation by calphostin C. C, Inhibition of FFA-induced Ser307 phosphorylation by PKC inhibitor.

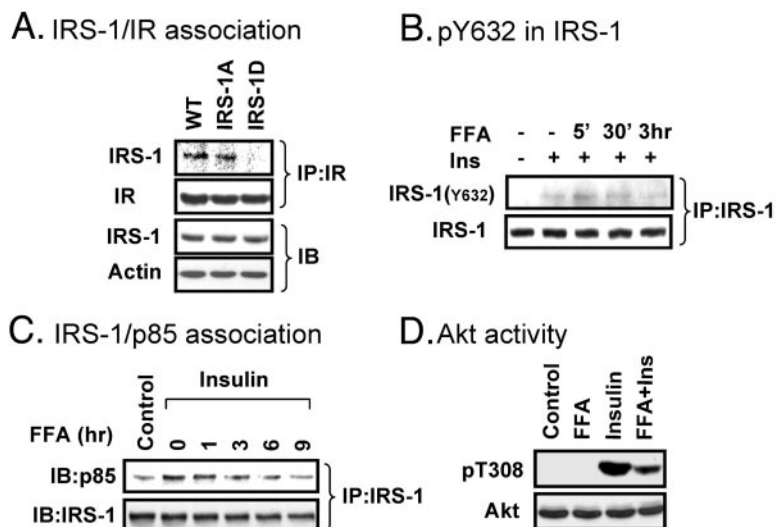


Fig. 6. Analysis of IRS-1 Function

IRS-1 function was examined by determining inhibitory effect of FFA. IRS-1 was studied by analyzing association of IRS-1/IR, IRS-1/p85, and by IRS-1 tyrosine phosphorylation as well as Akt phosphorylation. A, Reduction of IRS-1/IR interaction by Ser307 phosphorylation. NIH-3T3 cells that express insulin receptor were used in the study to host mutated IRS-1. IP was conducted with insulin receptor (IR) antibody. The IP product and recombinant IRS-1 in the whole cell lysate were blotted with IRS-1 antibody. B, Insulin-induced IRS-1 tyrosine phosphorylation. IRS-1 was immunoprecipitated from insulin-treated 3T3-L1 adipocytes. C, IRS-1 association with p85 of PI(3)K. 3T3-L1 adipocytes were used in IP with IRS-1 antibody. D, Akt activation by insulin. Akt phosphorylation at Thr308 was determined in 3T3-L1 adipocytes after 16-h treatment with linoleic acid. Insulin was used at 100 nM for 30 min.

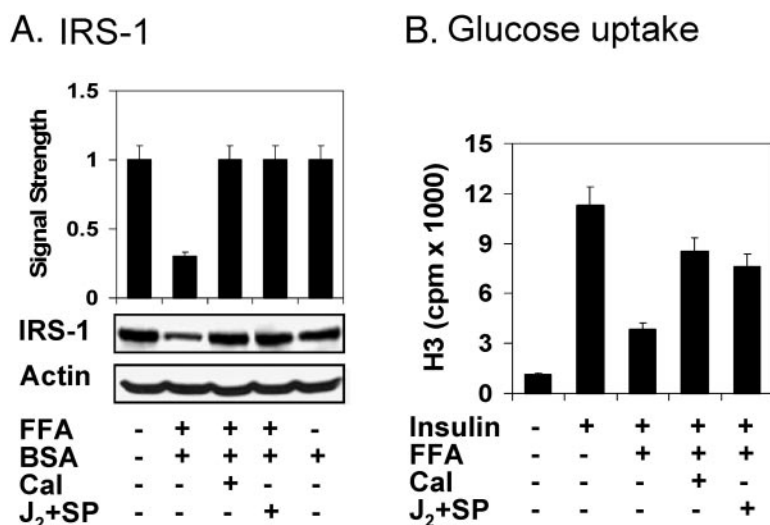


Fig. 7. Rescuing Glucose Uptake and IRS-1 Protein

Fully differentiated 3T3-L1 adipocytes were pretreated with calphostin C (Cal) or combination of 15dPGJ₂ (J₂) and SP600125 (SP) for 30 min, then followed by treatment of BSA-bound linoleic acid (300 μ M) for 16 h. IRS-1 protein abundance was examined in Western blot and glucose uptake was measured by H³-2D-glucose intake. A, Blocking FFA-induced IRS-1 degradation in 3T3-L1 adipocytes. Each bar represents means \pm SE of results from three experiments. A representative blot is shown. B, Insulin-induced glucose uptake was restored partially by the inhibitors. Each bar represents means \pm SE of results from triplicates. The experiment was repeated three times with consistent results.

dress this question, we examined PKC θ in the fat tissue of dietary obese mice. C57BL/6J mice were fed a high-fat diet to induce dietary insulin resistance. This insulin resistance is associated with body weight gain

in the mice (Fig. 8A). As markers of insulin resistance, hyperglycemia and hyperinsulinemia were detected at 8–10 wk on high-fat diet (Fig. 8, B and C). The PKC θ protein level was increased dramatically in the adipose

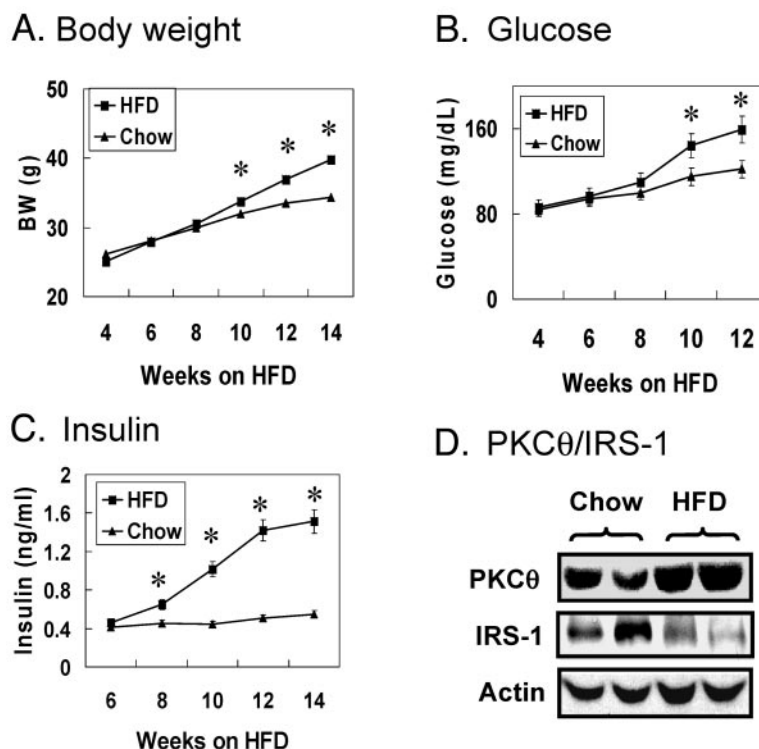


Fig. 8. PKC θ in the Adipose Tissue

C57BL/6J mice were fed a high-fat diet (HFD). Chow diet was used as a control. Body weight, fasting glucose, and insulin were measured weekly or biweekly. In this experiment, ten mice were examined in each group. *, Significant difference ($P < 0.05$). A, Body weight gain. B, Fasting glucose. C, Fasting insulin. D, Immunoblotting of PKC θ in the adipose tissue of normal and insulin-resistant C57BL/6J mouse. PKC θ protein level was determined in the whole cell lysate of fat tissue. The representative blot of two mice in each group is presented.

tissue when insulin resistance occurs in C57BL/6J mice on the high-fat diet (Fig. 8D), suggesting a chronic activation of the PKC θ serine kinase. Accordingly, the IRS-1 protein abundance was reduced in the adipose tissue of the dietary insulin-resistant mice (Fig. 7D). This observation is similar to PKC ϵ increase in the red muscles from fat-fed rats (26). These data suggest that hyperlipidemia activates PKC θ in fat tissue.

DISCUSSION

Mice deficient in IKK or JNK are protected from insulin resistance induced by the high-fat diet (37, 38). This information suggests that IKK or JNK may be involved in FFAs signal transduction that leads to insulin resistance. However, the activities of the two serine kinases have not been previously characterized in the FFA signaling at the cellular and molecular levels. This study provides evidence that IKK and JNK may mediate PKC signals for insulin resistance induced by FFAs in adipocytes.

IKK and JNK may contribute to IRS-1 serine phosphorylation in response to FFAs. In this study, we observed that both IKK and JNK were activated by linoleic acid, a FFA used in this study. The activation is

associated with the Ser307 phosphorylation in IRS-1. Inhibition of the two serine kinases led to protection from Ser307 phosphorylation and degradation of IRS-1 (Figs. 3 and 6). It is known that serine phosphorylation is associated with protein degradation of IRS-1 (16, 39). Because there are about 50 serine/threonine residues in IRS-1, it is hard to determine which serine/threonine is involved in the IRS-1 degradation. Recently, it has been shown that Ser307 phosphorylation contributes to IRS-1 degradation in hepatocytes in the response to insulin (14). Our result suggests that the same mechanism contributes to FFA-induced degradation of IRS-1 in adipocytes (Fig. 1). A decrease in IRS-1 abundance leads to insulin resistance as shown in IRS-1 knockout studies (40, 41). We observed that IRS-1 protein was reduced in the fat tissue of mouse model of dietary insulin resistance (Fig. 8B). This is consistent with that IRS-1 protein is reduced in the fat tissue of type 2 diabetes patient (42). We observed that inhibition of IKK and JNK by pharmacological agents was able to protect 3T3-L1 adipocytes from insulin resistance (Fig. 6). Taken together, our data support that IKK and JNK involve in FFA signaling pathway for insulin resistance.

IKK and JNK may mediate PKC signal for insulin resistance. It was reported that activities classical

PKCs and novel PKCs are negatively associated with insulin sensitivity (7, 24, 43, 44). Some reports suggest that activation of these two classes of PKCs by phorbol esters leads to activation of PI(3)K and glucose transporters (45, 46). Serine phosphorylation of IRS-1 represents a mechanism by which PKCs leads to the inhibition of insulin sensitivity (7, 35, 47, 48). However, it is not well defined how PKC promotes IRS-1 serine phosphorylation. PKC was shown to phosphorylate IRS-1 protein directly (49, 50); however, it remains to be established how PKC leads to Ser307 phosphorylation (32). In this study, we provide evidence that IKK and JNK may mediate PKC activity for Ser307 phosphorylation. The evidence includes: 1) FFAs induced activation of PKC θ as indicated by their phosphorylation status (Fig. 4); 2) Inhibition of PKC activities by specific inhibitor calphostin C resulted in suppression of both IKK and JNK activities (Fig. 5). The inhibition is associated with a reduction in IRS-1 serine phosphorylation (Ser307). Activation of IKK and JNK by PKC has been well established in the signaling pathways of cell membrane receptors. In B or T cells, PKC β and PKC θ are responsible for IKK and JNK activation, respectively. These have been demonstrated in signaling pathway of B-cell receptor and T-cell receptor (51, 52). Because IKK and JNK may act as downstream signal mediators for PKC, our data suggest that IKK and JNK mediate PKC θ signals in adipocytes.

PKC θ may be involved in the FFAs signaling pathway in adipocytes. It has been suggested that PKC θ is a major PKC isoenzyme in the skeletal muscle and activation of PKC θ by FFAs might be responsible for insulin resistance in the skeletal muscle (7, 24). Although PKC θ has drawn a lot of attention in the skeletal muscle (7, 24), it is not clear whether PKC θ plays a role in the adipose tissue. In this study, we evaluated PKC θ activity in adipocytes. Our result suggests that PKC θ is expressed in adipocytes and its phosphorylation is induced by FFAs (Fig. 4). In addition, PKC θ abundance is increased in the adipose tissue of dietary obese mice, suggesting a chronic activation of PKC θ . In addition to PKC θ , it was reported that PKC β (25), PKC δ (25), and PKC ϵ (26) could be activated by FFAs. However, these observations were made in muscle. In this study, our data suggest that PKC θ , but not other PKC isoforms, is activated by FFA in adipocytes (Fig. 4). This result suggests a tissue-specific effect of FFA activity.

We observed that phosphorylation of PKC can be induced in adipocytes by FFAs. It is generally believed that PKCs are constitutively phosphorylated at the activation and autophosphorylation domains in cells. However, it is not clear what is responsible for the constitutive phosphorylation. Our data suggest that the phosphorylation in some PKC isoforms is inducible by insulin. In serum-starved 3T3-L1 adipocytes, phosphorylation of PKC θ (Thr538), PKC δ (643), and PKC ζ / λ (Thr410/403) are induced by insulin (data not shown). Thus, insulin may be responsible for the constitutive phosphorylation of certain PKCs in cultured

cells that are maintained in serum-containing medium. Our observation is consistent with that insulin induces membrane association of PKC in 3T3-L1 adipocytes in serum-free condition (53). Because activities of most PKC isoforms are negatively associated with insulin sensitivity in cells and in animals as shown in PKC α or β knockout mice (44, 54), it is possible that activation of PKCs involves in the negative feedback of insulin signaling. In this study, we observed that phosphorylation of PKCs was induced by FFA in serum-free condition. It is possible that FFAs contribute to insulin resistance through activation of this negative feedback mechanism. An increase in intracellular diacylglycerol was suggested to contribute to PKC θ activation (7).

In summary, our data suggest a signaling pathway of FFAs for insulin resistance in adipocytes (Fig. 9). In this pathway, FFAs activate PKC isoenzymes such as PKC θ and leads to the activation of IKK and JNK. Activation of these two serine kinases leads to Ser307 phosphorylation in IRS-1. The serine phosphorylation is responsible for a reduction in IRS-1 protein and insulin resistance in adipocytes. This molecular pathway might operate in many cell types including adipocytes, myocytes, and hepatocytes.

MATERIALS AND METHODS

Reagents

Antibodies against phospho-IRS-1 (Ser³⁰⁷) (catalog no. 07-247) was obtained from Upstate Biotechnology (Lake Placid,

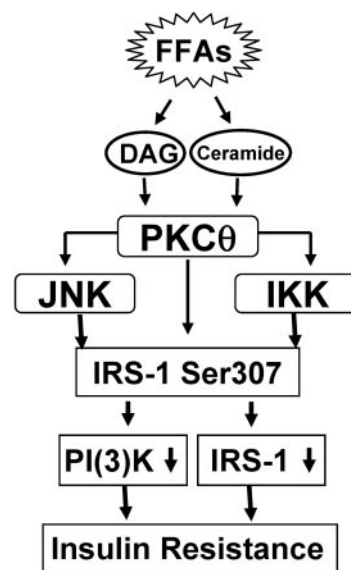


Fig. 9. Signal Transduction Pathway for Inhibition of IRS-1 Function by FFA

FFA activates PKC θ through diacylglycerol (DAG) or Ceramide. Then, PKC θ activates JNK and IKK for IRS-1 phosphorylation at Ser307. A reduction of IRS-1 function and protein abundance contributes to insulin resistance.

NY). Antibodies against IRS-1 (sc-7200), IRS-2 (sc-8299), GLUT4 (sc-7938), PI (3p85 (sc-423), insulin receptor β (sc-09), and phospho-JNK (sc-6254) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). β -Actin (ab6276) and GLUT1 (ab1932–125) antibodies were obtained from Abcam (Cambridge, UK). Phospho-specific antibodies to PKC α/β II (catalog no. 9375), PKC δ (catalog nos. 9374, 9376), PKC μ (catalog no. 2064), PKC θ (catalog no. 9377), PKC ζ (catalog no. 9378) and Akt (catalog no. 9275) were purchased from Cell Signaling Technology (Beverly, MA). Expression vectors for HA-IRS-1, HA-IRS-1A, and HA-IRS-1D were used in our previous study (11). JNK inhibitor SP600125 (catalog no. EI-305) was from Biomol (Plymouth Meeting, PA). 15dPGJ2 (catalog no. 538927) was from Calbiochem (San Diego, CA).

Dietary Obese Mice

Male C57BL/6J mice at age of 4 wk were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility at the Pennington Biomedical Research Center with 12-h light, 12-h dark cycle and constant temperature (23 C). The mice were free to access water and diet. After a 1-wk quarantine, the mice were divided into two groups, 12 mice per group. The experimental group was fed with high-fat diet (D12331, Research Diets, New Brunswick, NJ) in which fat accounts for 58 kcal%. The control group was fed with chow diet. All procedures were performed in accordance with National Institute of Health guidelines for the care and use of animal and approved by the Institute Animal Care and Use Committee at the Pennington Biomedical Research Center.

Fasting Plasma Glucose and Insulin

Fasting glucose and insulin were determined in the plasma every 2 wk. The blood (30 μ l/mouse) was collected from the tail vein using heparinized micro-hematocrit capillary tubes (catalog no. 22–362–566; Fisher Scientific, Pittsburgh, PA) after overnight (16 h) starvation. The plasma was prepared by centrifuging the blood at 4 C, 4000 rpm for 20 min. The glucose level was determined with a FreeStyle blood glucose monitoring system (TheraSense, Phoenix, AZ). The insulin level was determined with ELISA using the “Ultra Sensitive Insulin ELISA Kit” (catalog no. 90060, Crystal Chem, Chicago, IL).

3T3-L1 Adipocytes

The mouse fibroblast 3T3-L1 preadipocytes (CL-173) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM culture medium supplemented with 10% fetal calf serum, and 4 mM glutamine. For adipogenesis, 3T3-L1 preadipocytes were grown into confluence in a six-well or 100-mm plate, and then were differentiated into adipocytes using a standard protocol. The 3T3-L1 cells were incubated in the adipogenic cocktail (5 μ g/ml insulin, 0.5 mM isobutylmethylxanthine, and 10 μ M dexamethasone) for 2 d. This was followed by incubation in insulin-supplemented medium for additional 4 d. The normal medium was used at d 7 to maintain the adipocytes.

Fatty Acid Treatment

Stearic (S4751) was from Sigma (St. Louis, MO). Oleic (90260) and linoleic acids (90150) were purchased from Cayman Chemical (Ann Arbor, MI). These FFAs were mixed with FFA-free BSA (152401, ICN Biomedicals, Irvine, CA) at a weight ratio of 1:1 to make BSA-bound FFA. The 3T3-L1 adipocytes were serum-starved overnight in 0.1% BSA DMEM and then treated with BSA-bound FFAs.

Glucose Uptake (55)

3T3-L1 preadipocytes (5×10^5 /well) were differentiated into adipocytes in a 12-well plate. After serum-starvation in 0.1% BSA DMEM for overnight, the cells were incubated in 1 ml/well PBS containing 200 nM insulin for 30 min at 37 C. After washing in PBS, the cells were incubated in 1 ml PBS containing 0.1 mM 2-deoxyglucose and 1 μ Ci/ml 2-deoxy-D-[3 H] glucose for 5 min. Then, the cells were washed three times in ice-cold PBS, and solubilized in 0.4 ml of 1% sodium dodecyl sulfate. 3 H-glucose uptake was detected in 4 ml of scintillant using a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Nonspecific deoxyglucose uptake is measured in the presence of 20 μ M cytochalasin B and is subtracted from the total uptake to get specific glucose uptake.

Immunoblotting and IP (11)

The whole cell lysate was made by sonication in lysis buffer [1% Triton X-100, 50 mM KCl, 25 mM HEPES (pH 7.8), leupeptin 10 μ g/ml, aprotinin 20 μ g/ml, 125 μ M dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate]. IP was conducted with 200–400 μ g protein and 2–4 μ g antibodies. The IP product was then subjected to immunoblotting analysis. The protein was resolved in SDS-PAGE, and transferred onto polyvinylidene difluoride membrane (162–0184, Bio-Rad, Hercules, CA). The membrane was preblotted in milk buffer for 20 min, and then immunoblotted with a primary antibody for 1–24 h followed by a secondary antibody for 30 min. Horseradish peroxidase-conjugated secondary antibodies (NA934V or NA931, Amersham Life Science, Piscataway, NJ) were used with chemiluminescence reagent for signal imaging (NEL-105, PerkinElmer, Boston, MA). To detect multiple signals from a single membrane, the blot membrane was treated with a stripping buffer (59 mM trizma hydrochloride, 2% sodium dodecyl sulfate, 0.75% 2-merthylethylenediamine) for 30 min at 42 C, washed extensively in PBS for 2 h, and then used for reblotting with a different primary antibody. The intensity of Western blot signal was quantified with an image analysis program PDQuest 7.1 (Bio-Rad), and the signal was normalized against loading control.

Data Analysis

The data of glucose, insulin, glucose uptake and signals in immunoblot are presented as mean \pm SE of triplicates in a representative experiments or results of three independent experiments. Student's *t* test was used with significance of *P* < 0.05.

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